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SPECIAL REPORT

CB_1 receptor antagonist SR141716A increases capsaicin-evoked release of Substance P from the adult mouse spinal cord

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Cannabinoids have an antinociceptive action in many pain models. We have investigated a possible modulatory role for Type 1 Cannabinoid receptors (CB_1) on the release of excitatory transmitter Substance P from the adult mouse spinal cord after stimulation of nociceptor terminals by capsaicin. Capsaicin (0.1–10 μM) was applied to superfused cord sections and evoked a dose dependent release of SP above basal outflow of (23.36 ± 2.96 fmol 8 ml^{-1}). Maximum evoked SP release was obtained with 5 μM Capsaicin (262.4 ± 20.8 fmol 8 ml^{-1}). Higher capsaicin concentrations (50–100 μM) evoked less SP release. Superfusion of CB_1 antagonist SR141716A (5 μM) increased evoked SP release with capsaicin (0.1–10 μM) and reversed the reducing effect of high dose capsaicin (100 μM). Antagonism of CB_1 receptors in the spinal cord during capsaicin stimulation, is evidence of tonic CB_1 activity inhibiting the release of excitatory transmitters after activation of nociceptive neurones and is also indicative of endocannabinoid production during noxious stimulation.
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Abbreviations: Anandamide, arachidonylethanolamide; Ca^{2+}, Calcium ions; CB_1, Cannabinoid receptor type1; CGRP, calcitonin gene related peptide; SP, Substance P; SR1 & SR141716A, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]; VR1, vanilloid receptor.

Introduction The antinociceptive effect of cannabinoids exogenously applied in various models of acute and tonic pain, may be partly explained by the abundance of CB_1 receptors in the superficial laminae of the spinal cord, where C and Aδ fibre nociceptors terminate. Many of these terminals contain the tachykinin peptide transmitter Substance P (SP), which is co-released with glutamate after noxious stimulation of nociceptive fibres. SP is an important component of the pain signal, especially after intense noxious stimulation. When released it acts synergistically with glutamate to excite post synaptic dorsal horn neurones (reviewed Hökfelt *et al*., 2001). CB_1 is found at two cellular locations in the superficial spinal cord, pre-synaptically on primary afferent terminals (Hohmann & Herkenham, 1999; Ahluwalia *et al*., 2000) and on a population of excitatory interneurons expressing the gamma sub-unit of protein Kinase C (Farquar-Smith *et al*., 2000). CB_1 receptors are coupled to G(i)/(o) proteins, whose actions tend to inhibit neuronal excitability by activation of hyperpolarizing potassium currents (Shen *et al*., 1996), or by inhibition of Ca^{2+} channels reducing intracellular Ca^{2+} levels in order to inhibit transmitter exocytosis (Robbe *et al*., 2001). CB_1 receptor activation has been shown to inhibit transmitter release from hippocampal circuits (Shen *et al*., 1996). The CB_1 agonist anandamide has been used to inhibit Ca^{2+} influx (Szóke *et al*., 2000) and the release of CGRP from rat sensory neurone terminals after capsaicin or electrical stimulation (Richardson *et al*., 1998, Tognetto *et al*., 2001). In this study, we aimed to investigate the endogenous activity of spinal cord CB_1 receptors and their influence on excitatory transmission from nociceptors after noxious stimulation.

Methods Spinal cords from adult CD1 mice (20–30 g Charles River U.K.) were extruded and 16 mm sections mounted then sealed in a chamber continuously superfused with oxygenated (5% CO_2) Krebs' solution (1 ml min^{-1}) containing (in mM): (NaCl; 118, KCl; 4, $MgSO_4$; 1.2, KH_2PO_4; 1.2, $NaHCO_3$; 25, $CaCl_2$; 2.5 and glucose; 11). Krebs' solution was supplemented with 0.1% bovine serum albumin, phosphoramidon 1 μM, captopril 100 μM, dithiothreitol 6 μM, bacitracin 20 μg ml^{-1} (all from Sigma). Superfusate outflow from cord sections was sampled in a series of seven 8 ml fractions, each collected for 8 min at room temperature into glass tubes containing acetic acid (0.1 N). The first three fractions were collected in the absence of stimulation to determine basal SP outflow. Sections were stimulated during collection of the fourth fraction by superfusing capsaicin (0.01–100 μM) for 3 min. Three fractions were collected post-stimulation. Samples were concentrated and desalinated using Sep-Pak C_{18} cartridges (Waters, Watford, U.K.) then dried under nitrogen. SP-like immunoreactivity (SP-LI) of reconstituted samples was measured within the detection limits of a radioimmunoassay (1–200 fmol per assay tube, see Malcangio & Bowery, 1993).

Data analysis Effect of capsaicin on SP release was determined by statistical comparison of average SP content in each basal fraction *versus* content in fractions after capsaicin stimulation. The effects of drug superfusion in addition to capsaicin were also compared, (two-way (ANOVA) followed by Tukey test). Data were also expressed

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as SP content in the stimulated fraction corrected for average SP content in basal fractions for each cord section (evoked SP release). Estimated EC_{50} values were calculated using GraphPad Prism software.

Drugs Capsaicin, Capsazepine and Anandamide (from Sigma RBI), were dissolved in ethanol then diluted in Krebs' solution. SR141716A (a gift from Sanofi Recherche, Montpelier France to Dr S Paterson) was dissolved in 100% dimethyl sulphoxide (Sigma) then diluted in Krebs' solution.

Results *Capsaicin evoked release of SP from mouse spinal cord* Capsaicin superfusion significantly increases release of SP-L1 measured in superfusate outflow from mouse spinal cords, above basal non-stimulated levels (Figure 1). To verify that the capsaicin-evoked release was mediated by activation of the receptor for capsaicin (VR1), the competitive VR1 antagonist capsazepine (100 μM) was superfused 3 min before stimulation, co-superfused with capsaicin (10 μM) and superfused 2 min after stimulation. Capsazepine treatment significantly inhibited the release of SP-LI, with this concentration of capsaicin (Figure 1).

Using capsaicin concentrations 0.1–10 μM, the evoked release of SP-LI increased dose dependently with an estimated EC_{50} value of 311 nM (95% C.I., 178–542 nM) (Figure 2, solid line). Low doses of 0.01 μM capsaicin were ineffective at releasing SP-LI above basal outflow. Peak evoked release of SP-LI was achieved using 5 μM capsaicin. At higher capsaicin doses (50–100 μM), the amount of evoked SP-LI was markedly reduced, producing a bell shaped dose-response curve (Figure 2, dotted line).

CB_1 antagonist SR141716A modulates capsaicin-evoked SP-LI release Superfusion of the CB_1 antagonist SR141716A 5 μM (8 min pre-stimulation, 3 min co-stimulation with capsaicin and 13 min post-stimulation), significantly in-

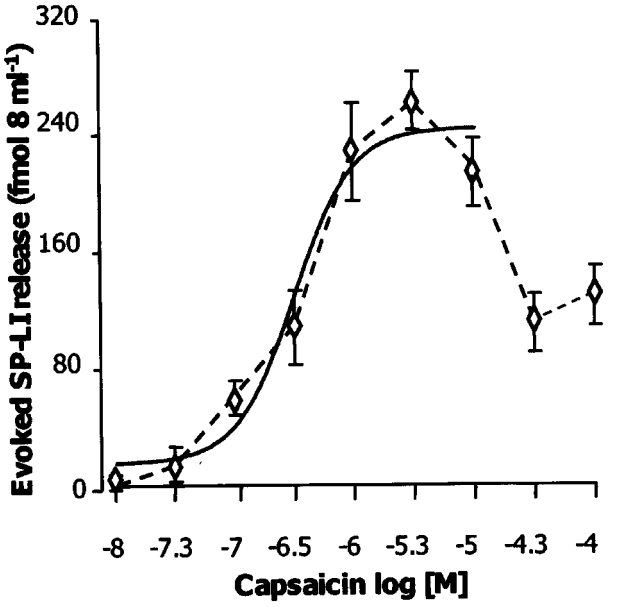

Figure 2 SP-LI release from cord sections evoked by capsaicin superfusion. Dotted line: Bell-shaped curve of mean±s.e.mean evoked SP-LI release with capsaicin (0.01–100 μM) after subtraction of mean basal outflow SP-LI (23.36±2.96 fmol 8 ml^{-1}), (n=3–5 per dose group). Solid line: Calculated SP-LI release dose response curve for capsaicin (0.01–10 μM).

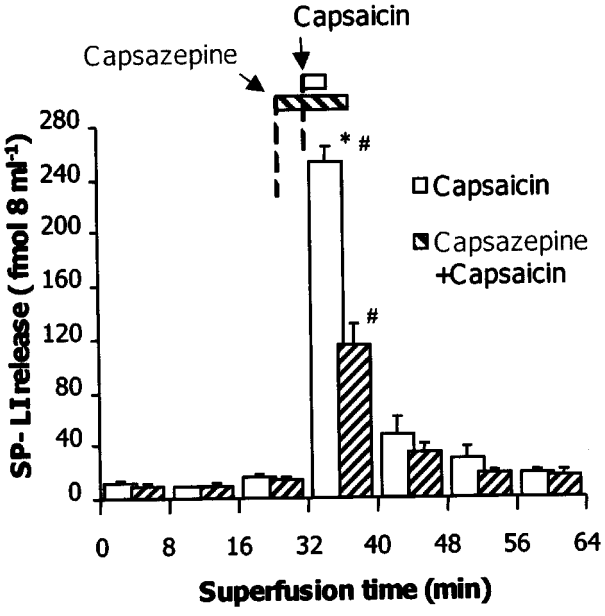

Figure 1 Mean SP-LI contents±s.e.mean in cord superfusates collected in 8 min fractions. In both groups capsaicin superfusion (10 μM) (white horizontal bar), during collection of the 32–40 min fraction, significantly increases release of SP-LI above the three basal fractions, #P<0.05 two-way (ANOVA) and Tukey test (n=6 per group). Capsazepine superfusion (100 μM) (hatched horizontal bar) produces a significant reduction in SP release after capsaicin stimulation, compared to controls *P<0.05 two-way (ANOVA) and Tukey test.

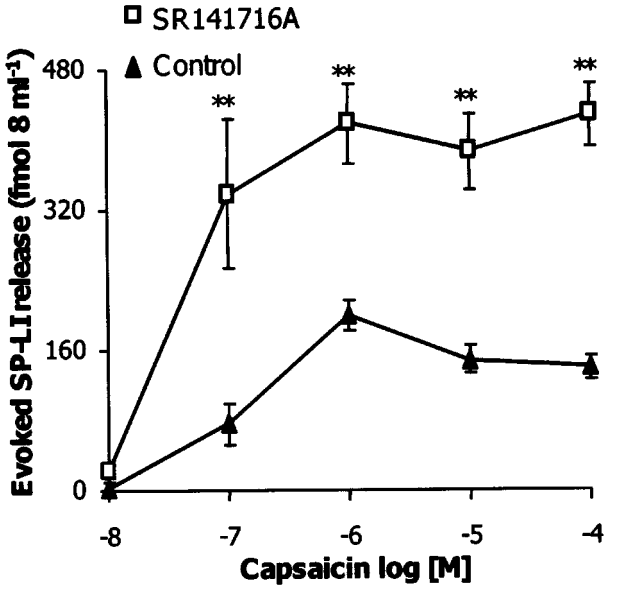

Figure 3 Capsaicin induced SP-LI release is increased by CB_1 antagonist SR141716A. Data represent mean±s.e.mean SP-LI release from mouse cords evoked by capsaicin superfusion, after subtraction of mean basal outflow from each cord (n=3–5 per treatment group). Superfusion of SR141716A significantly increased capsaicin (0.1–100 μM) evoked release compared to control slices superfused with capsaicin only. **P<0.001 Two-way (ANOVA) then Tukey test (mean evoked SP-LI release for control *versus* SR1 treated cords at each capsaicin dose).

creases the evoked release of SP-LI, obtained with capsaicin doses 0.1–100 μM (Figure 3). Following SR1 superfusion 8 min before stimulation, mean SP-LI release was (34.0 ± 5.84 fmol 8 ml^{-1}). SR1 failed to alter basal levels of SP-LI release significantly when compared to samples collected before SR1 superfusion (29.76 ± 5.44 fmol 8 ml^{-1}), ($n=21$ cord sections), nor did it significantly increase release from cords treated with the non-effective 0.01 μM concentration of capsaicin. After SR1 treatment, SP-LI release was maximized using the 100 μM capsaicin dose, instead of 5 μM. This suggests that the reduction in SP-LI release seen at high capsaicin doses, may be mediated by CB_1 activity.

CB_1 agonist Anandamide inhibits capsaicin-evoked SP-LI release CB_1 receptors in the spinal cord were activated by superfusion of a receptor agonist anandamide for 3 min before and during superfusion of capsaicin. Anandamide had no effect on basal SP-LI measured in 8 and 16 min samples, but significantly inhibited the release of SP-LI compared to control cords superfused with the same concentration of capsaicin (Figure 4).

Discussion In this study we show that the CB_1 receptor antagonist SR141716A modulates capsaicin stimulated release of the excitatory transmitter SP from the mouse spinal cord. Release levels evoked by capsaicin are comparable with stimulated SP release from rat dorsal horn preparations (Malcangio & Bowery, 1993). A significant proportion of evoked SP-LI release in mouse superfusates is attributable to a VR1 receptor mediated response to capsaicin, as SP-LI release is significantly reduced by the VR1 antagonist capsazepine. The effective capsaicin dose range used to release SP is also close to the reported 1 μM affinity for capsaicin at VR1 (Caterina *et al*., 1997). Electrophysiological studies indicate that most capsaicin sensitive neurones are polymodal nociceptors (Holzer, 1991) and most SP and CGRP containing cells of this population express mRNA for VR1 (Michael & Priestley, 1999). It is likely that most capsaicin-evoked SP is released from nociceptive neurones expressing VR1 as these neurones can release peptides after capsaicin stimulation 1–100 μM, even when isolated from other cell types in culture (Ulrich-Lai *et al*., 2001). Increases in capsaicin-stimulated release of SP in the presence of the CB_1 antagonist SR1, implies that CB_1 receptors are tonically active in the spinal cord and function to reduce SP release after stimulation of nociceptive fibres. Activating these receptors by exogenously applying the CB_1 agonist anandamide to spinal cord sections, reduces the capsaicin-evoked release of SP, as was previously shown for the release of CGRP peptide from the rat dorsal horn (Tognetto *et al*., 2001; Richardson *et al*., 1998). In addition, some behavioural studies have shown that intrathecal delivery of SR1 to the spinal cord lowers the nociceptive threshold in both normal and hyperalgesic animals (see Walker *et al*., 2000), suggesting tonic modulation of nociceptive responses by CB_1. In this study, SR1 does not increase either basal SP release or release with a non-effective capsaicin concentration (0.01 μM), suggesting that CB_1 activity is only effective in regulating SP release after sufficient nociceptor stimulation. The SR1 mediated increase in SP-LI, was not related to the effectiveness of the dose of capsaicin used to stimulate its release. This may be indicative of inverse agonist activity reported for SR1 (Walker *et al*., 2000). Alternatively, this could be explained by a non-specific action of capsaicin, such as activating depolarizing Potassium channels or desensitizing VR1 receptors (Bevan & Szolcsanyi, 1990). The reduction in SP release using high dose capsaicin appears to be CB_1 mediated, as this effect is reversed using SR1, perhaps reflecting that the endogenous activity of CB_1 receptors is increased after intense 'noxious' stimulation with high dose capsaicin. This could also be evidence that production of endogenous cannabinoids might be induced by noxious stimulation, in order to account for the increased activation of these receptors. Formation of the putative endogenous CB_1 ligand anandamide seems to occur after Ca^{2+} dependent depolarization of neurones (Di Marzo *et al*., 1993) and its noxious stimulus-evoked release has been demonstrated in periaquaductal grey tissue after formalin injections (Walker *et al*., 1999).

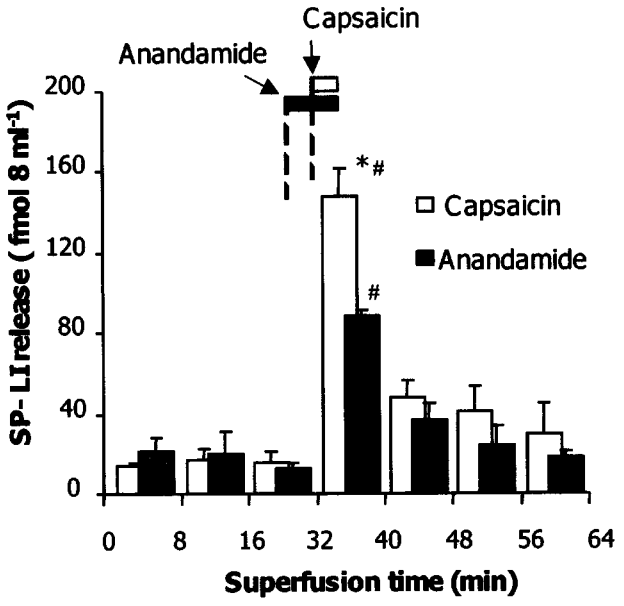

Figure 4 Mean SP-LI contents ± s.e.mean in cord superfusates collected in 8 ml fractions. In both groups capsaicin supersusion (300 nM) (white horizontal bar) during collection of the 32–40 min sample, significantly increases release of SP-LI above basal fractions, #$P<0.05$ two-way (ANOVA) and Tukey test ($n=5$ per group). Anandamide superfusion (1.44 nM) (grey horizontal bar), reduces SP-LI release after superfusion of capsaicin compared to control cords, *$P<0.05$ two-way ANOVA then Tukey test.

Anandamide has been recently detected in spinal cord tissue, but its release after stimulation has yet to be demonstrated (Di Marzo *et al*., 2000), however *in vitro* studies showed cells over-expressing VR1, released anandamide in response to capsaicin stimulation (Di Marzo *et al*., 2001). This study provides further evidence that the release of excitatory transmitters from stimulated nociceptor terminals in the spinal cord is tonically controlled by endogenous cannabinoids. The mechanism by which CB_1 executes this negative control depends on where cannabinoids are acting: by reducing Ca^{2+} dependent neurosecretion directly from their pre-synaptic receptors located on primary afferent terminals, where VR1 is often co-expressed (Ahluwalia *et al*., 2000), or indirectly at interneuronal CB_1 sites where they might act as retrograde messengers, reducing post-synaptic excitation levels in the cord, in order to prevent potentiation

of pre-synaptic transmitter release. This study supports a role for the endogenous cannabinoid signalling system in modulating nociceptive transmission in the spinal cord.

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